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Origins and consequences of mitochondrial decline in nucleated erythrocytes

C.D. Moyes*, M.L. Sharma, C. Lyons, S.C. Leary, M. Leon, A. Petrie, S.G. Lund, B.L. Tufts

Biosciences Complex, Department of Biology, Queen's University, Kingston, ON, Canada, K7L 3N6

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Abstract

Cellular aging in nucleated erythrocytes from lower vertebrates is accompanied by losses in mitochondria but it remains unclear (i) how these losses accrue (ii) if these changes alter energetics and (iii) whether such changes increase the propensity for apoptosis. We addressed these questions using trout erythrocytes that were separated into age classes using inherent differences in buoyant density. The oldest cells showed a profound decline in mtDNA transcripts, due to reductions in both transcription (90% decline in total RNA) and mtDNA copy number (35%). No alterations in the ratio of 16S rRNA to COX I mRNA were detected, nor was there an accumulation of unprocessed mtDNA transcripts. While older cells had reduced basal respiration, there were no changes in mitochondrial enzymes stoichiometries, tissue ATP levels or dinitrophenol-induced (maximal) respiration rates. Apoptosis could not be induced in either whole blood, young or old erythrocytes by pro-oxidants, mitochondrial inhibitors or staurosporine. In contrast, cyclosporin A (CsA) caused caspase 3 activation, DNA laddering and LDH leakage, but only in young cells. Both CsA and a combination of azide, oligomycin and dinitrophenol cause mitochondrial depolarization and caspase 9 activation, but only CsA induced caspase 3 and apoptosis. Caspase inhibitor studies support the conclusion that mitochondrial changes may accompany CsA-induced cell death, but are not essential in its progression. While pifithrin failed to induce cell death, it enhanced the effects of CsA, implicating a role for p53. Collectively, these studies suggest that the mitochondrial changes with aging do not compromise cellular function, although trout erythrocytes can initiate apoptosis by non-mitochondrial pathways.

Keywords: Apoptosis; Cyclosporin; Cytochrome-oxidase; Respiration; Oxidative phosphorylation

1. Introduction

Mitochondria have many roles in eukaryotic cell biology. Most of the energy required for cellular function is produced by mitochondria through oxidative phosphorylation (OXPHOS). The mitochondrial respiratory chain is also a primary source of reactive oxygen species (ROS), which can be both cytotoxic and regulatory. Mitochondria are also involved in apoptosis; the opening of the mitochondrial permeability transition pore (MPTP) leads to cytochrome *c* release, triggering cell death through the activation of caspase 9 [1,2]. Defects in mitochondria can therefore exert pleiotropic effects on cellular energetics, signal transduction and gene regulation. Although mitochondrial dysfunction has been implicated in numerous neurodegenerative and cardiovascular diseases [3], mitochondrial decay may be a normal element of aging in some cell types. Indeed, mitochondrial variation need not have negative consequences to the cell or organism [4].

Erythrocytes of lower vertebrates experience many structural and functional changes throughout their 4–6-month life span, including loss of mitochondria [5–13]. While there are reductions in biosynthetic processes and the ability to mount heat shock responses, erythrocytes of fish do not appear to lose the capacity to perform ion and gas transport as they age [12,13]. The mitochondrial losses that occur throughout their life could result from reduced synthesis (e.g., transcriptional down-regulation) or accelerated degradation (e.g., mitochondrial autophagy). Recent studies suggest cellular aging in fish erythrocytes is associated with declines in $\Delta\Psi$ [11], a general phenomenon in models of

Abbreviations: CS, citrate synthase; CsA, cyclosporin A; COX, cytochrome oxidase; G6PDH, glucose 6 phosphate dehydrogenase; HK, hexokinase; LDH, lactate dehydrogenase; MPTP, mitochondrial permeability transition pore; mtDNA, mitochondrial DNA; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; TMRM, tetramethylrhodamine-methyl ester

* Corresponding author. Tel.: +1-613-533-6157; fax: +1-613-533-6617.
E-mail address: moyesc@biology.queensu.ca (C.D. Moyes).

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cellular aging. Although nucleated erythrocytes rely upon OXPHOS for ATP synthesis [13], it is not known if these mitochondrial changes are problematic or simply reflect reduced energetic demands [12]. In addition to the potential impact of mitochondrial deterioration on energetics, the changes could make cells more sensitive to MPTP-initiated apoptosis. In nucleated erythrocytes of lower vertebrates, little is known about the relative importance of necrotic vs. apoptotic (either mitochondrial- or ligand-initiated) cell death. Immature mammalian erythrocytes are nucleated and undergo apoptosis in response to ligand interactions in the erythropoietic tissue [14], whereas enucleated mature erythrocytes are removed from the circulation by the immune system [15].

In the present study, we examined aging erythrocytes of rainbow trout to determine (i) the mechanisms by which mitochondrial losses accrue, and if these changes (ii) compromise bioenergetics or (iii) alter the propensity to undergo mitochondrial-induced apoptosis.

2. Materials and methods

2.1. Trout erythrocyte preparation and fractionation

Rainbow trout (*Oncorhynchus mykiss*) of undetermined sex were obtained from Pure Springs Trout Farm and maintained in tanks of dechlorinated water at 8–15 °C prior to sampling. Blood was collected from one to two large fish (500–1500 g) and fractionated using previously described techniques [12,13], modified from Speckner et al. [9]. As cells age, they accumulate hemoglobin and increase in density. Age-dependent differences in density can be used to separate cells by self-sorting [9,12,13] or through the use of Percoll gradient-based separations [10].

2.2. Construction of cDNA probes

Preparation of rainbow trout-specific probes has been described previously [16]. New probes were synthesized using polymerase chain reaction (PCR) and specific primers based upon the trout mtDNA sequence (GenBank accession #L29771). The mtDNA probe spanned the COX II–COX III genes, with primers flanking the region from 8181 to 10555 (forward: 5′-atggcacatccctcacaact-3′, reverse: 5′-gcctcatcagtaaatagaga-3′). The probe for unprocessed transcript spanned the genes for the serine and aspartate tRNAs with primers flanking the region from 8019 to 8166 (forward: 5′-taagacacgcggggtttaaac-3′, reverse: 5′-cgagaaagggaggaattgaa-3′). The amplification reactions contained 1 μl of trout DNA, 5 μl of reaction buffer, 1.5 mM $MgCl_2$, 1 μl 10 mM dNTPs, 250 ng of the specific primer and 0.3 μl Taq polymerase in autoclaved water to a total of 50 μl. Amplification consisted of 30 cycles of denaturation at 95 °C (15 s), annealing at 54 °C (60 s), extension at 72 °C (60 s), and a final extension at 72 °C (10 min). PCR products were separated on a 0.8% (mtDNA probe) or 2% (unprocessed transcript probe) agarose gel stained with 0.5 μg/ml ethidium bromide, excised and purified with Qiagen's Qiaex II gel purification kit. Double-stranded probes (50 ng) were labelled using Ready-to-Go Beads (Pharmacia) and 50 μCi of ^{32}P-α-dCTP isotope (3000 mCi/mmol). The probe for mtDNA was labelled using specific primers to generate full-length probe. The probe for unprocessed transcript was a single-stranded anti-sense cDNA probe complementary to a pair of tandem tRNAs. For the latter two probes, 50 ng template was combined with 200 ng primers (for unprocessed transcript, only the reverse primer was used) in 14 μl. The mixture was then denatured and combined with 2.5 μl 0.5 mM dNTPs (minus dCTP), 2.5 μl (10×) Klenow fragment buffer, 1 μl of Klenow fragment and 50 μCi of ^{32}P-α-dCTP (3000 mCi/mmol) and incubated for 12–16 h at room temperature. In all cases, probes were purified on G-50 Sephadex columns and typically demonstrated 60–80% radionucleotide incorporation.

2.3. DNA isolation, quantification and analysis

Total cellular DNA was purified from fractionated blood (50 μl) using proteinase K digestion and phenol/chloroform purification as previously described [16]. Slot blots of total cellular DNA were prepared by diluting 10 μg/fraction of cellular DNA to 200 μl in a solution of autoclaved water, 0.5 M NaCl and 0.25 N NaOH. Samples were vortexed, denatured in boiling water for 10 min and cooled on ice. An equal volume of 0.25× SSC was added and samples were carefully placed in the loading platform above a Duralon nylon membrane pre-wetted with 10× SSC. Samples were drawn through the wells using vacuum pressure and washed with 1 ml of 6× SSC. The membrane was then air-dried for 30 min and UV cross-linked to fix the DNA to the membrane.

Slot blots were prehybridized for 30 min at 65 °C in a Hybaid mini-hybridization oven with a 12 ml solution of QuickHyb prior to addition of labelled mtDNA probe. After 6 h of hybridization at 65 °C, the membrane was washed for 15 min with 50 ml of solution A (2× SSC, 0.1% SDS) at 42 °C, B (1× SSC, 0.1% SDS) at 42 °C, C (0.25× SSC, 0.1% SDS) at 42 °C then D (0.1× SSC, 0.1% SDS) at 65 °C. Transcript levels of mtDNA were quantified using a Molecular Dynamics Phosphoimager and Image Quant software.

2.4. RNA isolation, quantification and analysis

Total cellular RNA was obtained from fractionated blood using acid-phenol extraction as described previously [16]. Purified RNA was glyoxylated, electrophoresed (1% agarose) and transferred to nylon as previously described. Northern blots were prehybridized with 12–15 ml of solution (25 mM KH_2PO_4 at pH 7.4, 5× SSC, 5× Denhardt's, 50 μg/ml salmon sperm DNA and 50% for-

mamide) at 42 °C. Hybridization solution (prehybridization solution with 0.2 M dextran sulfate) containing the COX I probe (denatured and labelled) was added and hybridization continued for 18 h at 42 °C. The membrane was then washed under the same conditions as the slot blots and the transcripts quantified by phosphoimaging.

2.5. Cellular respiration

Respiration experiments were run in water-jacketed vessels fitted with YSI 53310 oxygen electrodes at 13 °C. Erythrocytes were prepared to 10% hematocrit in phosphate-buffered saline, incubated for 30 min at 13 °C and added to the chamber. To induce maximal (uncoupled) respiration, cells were treated with optimal concentrations of dinitrophenol (0.35 mM). Basal and uncoupled respiration rates in fraction 1 and 6 cells were recorded separately for 30–60 min for each treatment. The oxygen consumption within each chamber was monitored using LoggerPro software.

2.6. Adenylates

Adenylates were assayed using acid extracts of freshly fractionated erythrocytes using enzyme-linked assays. Cells (400 μl) were acidified with 400 μl of 6% perchloric acid, shaken and centrifuged at 10,000 × *g* for 1 min. The supernatant was removed and recentrifuged. Samples were neutralized (pH 7.0–7.5) using 100 μl of 2 M KCl, 50 μl of saturated Tris base and 10–20 μl of 1 M KOH, then chilled on ice and centrifuged (5 min at 12,000 × *g*). ADP was assayed using pyruvate kinase and lactate dehydrogenase. ATP was assayed using hexokinase and glucose-6-phosphate dehydrogenase.

2.7. Enzyme assays

Lysates were prepared by combining 500 μl erythrocytes with 6 ml of deionized water. After 30 s of gentle agitation, an equal volume of 280 mM KCl–40 mM HEPES (pH 7.5) was added. This lysate was used directly to measure whole tissue levels of several enzymes. Lysates were also used to prepare a mitochondrial pellet. Lysates were centrifuged at 4 °C for 10 min at 1000 × *g*. The supernatant was centrifuged for 10 min at 10,000 × *g*. The resulting mitochondrial pellet was washed in a buffer containing 140 mM KCl and 20 mM HEPES (pH 7.5) and recentrifuged. Mitochondrial pellets were resuspended in cell extraction buffer (20 mM HEPES, 0.1% Triton X 100 and 1 mM EDTA, pH 7.4). All assays were performed at 25 °C in triplicate using a Molecular Devices Spectramax Plus spectrophotometer. This assay temperature, although close to the upper thermal limit of this species, resulted in linear rates of enzyme activity during the duration of the assay. Mitochondrial extracts were used to measure citrate synthase (CS, EC 4.1.3.7), complex I (EC 1.6.5.3) and cytochrome oxidase (COX, EC 1.9.3.1) as previously described [17].

2.8. Apoptosis

In an attempt to induce apoptotic death in erythrocytes, cells were incubated for up to 48 h with combinations of chemicals known to influence apoptosis. Erythrocytes were suspended in a solution of sterile Hepes-buffered DMEM with penicillin–streptomycin–neomycin to a final volume of 500 μl each, at 10% hematocrit and maintained at 10 °C. DMEM was used over saline to provide nutrients that could improve cell survival during long incubations. Hepes buffer was chosen over bicarbonate–CO_2 because water breathers have very low pCO_2. Treatments included azide (10 mM), cyanide (10 mM), oligomycin (2.4 μg/ml), dinitrophenol (0.35 mM), sodium nitroprusside (10 mM), paraquat (0.5 mM), staurosporine (2–5 μM), actinomycin D (20 μg/ml), as well as their respective vehicles (DMSO or ethanol). Preliminary studies revealed cyclosporin A (CsA) at high concentrations induced apoptosis. Negligible cell death occurred at concentrations below 25 μM. Maximal effects were seen at CsA concentrations of 100–250 μM. Since CsA typically exerts its effects through interactions with cyclophilins, a series of studies using other inhibitors of cyclophilin-dependent processes was conducted. Pifithrin (200 μM), an inhibitor of p53, and FK506 (1 nM–1 μM), an inhibitor of calcineurin, were also employed. Cells treated with CsA (250 μM) were co-incubated with one of a set of caspase inhibitors (R&D Systems), each at 50 μM. Fluorescent substrates were used to measure caspase 3 (Ac-DEVD-AMC) and caspase 9 (Ac-LEHD-AFC) activities (Calbiochem).

After treatment with pro- or anti-apoptotic factors, erythrocytes were assayed for leakage of LDH (EC 1.1.1.27) and nuclear fragmentation. Samples were centrifuged at 1800 × *g* for 10 min. The supernatant (5–50 μl) was assayed for LDH using 1 mM pyruvate and 0.15 mM of NADH in 20 mM HEPES buffer (pH 7.0), along with 5–50 μl of supernatant or lysed cell pellet. Samples were read at 340 nm for 5 min and assayed in triplicate. The percentage of LDH in the supernatant vs. total LDH in the cell and supernatant indicated the effectiveness of the treatment in causing membrane breakage and thus, cell death (either necrotic or apoptotic). In nuclear isolations, erythrocytes were resuspended in 500 μl of 10 mM HEPES, 10 mM KCl, 2 mM $MgCl_2$ (pH 7.4) and left on ice for 5–10 min to fully lyse. The solution was layered onto 5 ml of the same buffer containing 30% sucrose, then centrifuged at 3000 × *g* for 10 min. Both layers of the supernatant were aspirated, and the resulting nuclear pellet digested for 18 h at 50 °C with proteinase K digestion buffer as previously described in Section 2.3. Samples (1–10 μg) were electrophoresed on a 2% gel at 87 V and post-stained with ethidium bromide.

2.9. Fluorescence microscopy

Red blood cells (1 μl/ml) were incubated at 15 °C for 30 min with 15 nM tetramethyl rhodamine, methyl ester

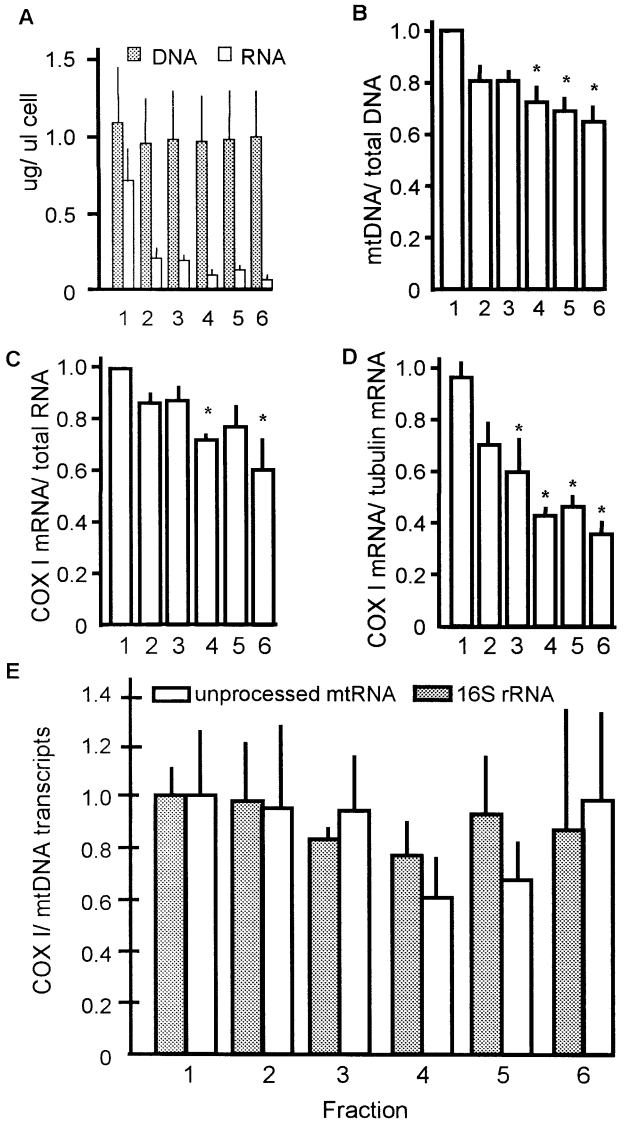

Fig. 1. Changes in RNA, DNA and mitochondrial DNA transcript levels in aging trout erythrocytes. Fraction 1 represents the youngest cells, fraction 6 the oldest ($n=7$). Panel A represents measurements of RNA (open bars) and DNA (closed bars) yield from cell extractions. Panel B summarizes the results from slot blot analysis of mtDNA in 5 or 10 μg total DNA. COX I mRNA levels were determined from Northern blots of 10–20 μg total RNA ($n=5$). Results are expressed relative to total RNA (Panel C) and tubulin mRNA (Panel D). The same blots were probed for levels of other mtDNA transcripts including 16S rRNA (solid bars) and an unprocessed intermediate transcript (open bars) (Panel E). Columns denoted with * are significantly different from fraction 1 (Tukey–Kramer, $p<0.05$).

(TMRM, Molecular Probes) on a poly-L-lysine-treated glass cover slip. Treatments included either staurosporine (5 μM) or a combination of azide (10 mM), dinitrophenol (0.35 mM) and oligomycin (2.4 μg/ml). After incubation, the cover slip was washed with clear DMEM, placed on a drop slide and viewed immediately under a Zeiss deconvoluting fluorescent microscope. Photos were taken using a CCD camera (Cooke Sensicam) with 100 ms exposure time (2 × 2 binning), utilizing the appropriate filter sets for TMRM fluorescence (excitation = 548 nm, emission = 573 nm). Using the Slidebook analysis software package (Intelligent Imaging Innovations), the pixel histogram for each image was set to a fixed range in order to show the qualitative differences in mitochondrial fluorescence between treatments.

2.10. Statistical analysis

Significant differences ($p<0.05$) between fractions were determined using a one-way analysis of variance (ANOVA), with post hoc analysis determined by the Tukey–Kramer

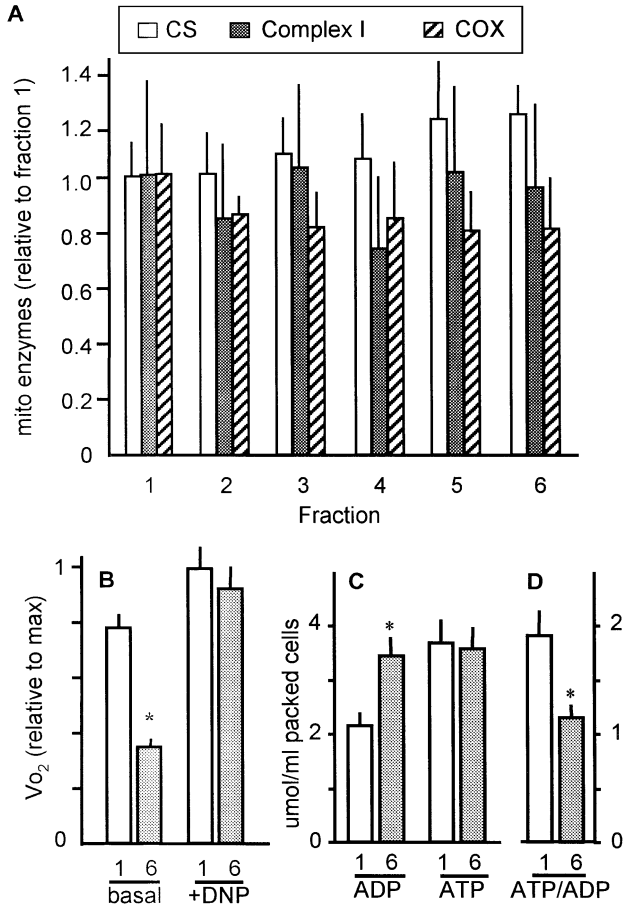

Fig. 2. Changes in mitochondrial functional parameters with cellular aging. Panel A summarizes the relative levels of mitochondrial enzymes measured in mitochondrial fractions. Fraction 1 relative activity corresponds to 0.49 ± 0.06 (CS, $n=12$), 0.074 ± 0.025 (complex I, $n=6$) and 0.12 ± 0.021 (COX, $n=7$). Activities are nmol product formed or substrate lost/μg mitochondrial protein/min. No significant differences exist between fractions (ANOVA, $p>0.05$). Panel B. Respiration rates were determined for cells that were either untreated (basal) or treated with 0.35 mM dinitrophenol (uncoupled) to elicit maximal respiration rates ($n=7$). Fraction 1 rate corresponds to 1.75 ± 0.10 (basal), and 2.25 ± 0.17 (uncoupled) nmol O_2 consumed/μl erythrocyte/min. Panel C. Adenylate content of cells ($n=3$). Adenylate contents are nmol of ADP or ATP/μl erythrocyte. Columns denoted with * represent significant difference from fraction 1 (paired t-test, $p<0.05$).

test. When only fractions 1 and 6 were compared, we used paired *t*-tests ($p < 0.05$).

3. Results

3.1. Mitochondrial gene expression

Analysis of age-dependent mRNA changes is complicated by the profound reduction in total RNA levels. Although total DNA levels remain constant, total RNA levels dropped by 90% (Fig. 1A). The levels of mtDNA showed a 35% decline with cell age (Fig. 1B). Northern analysis revealed COX I mRNA levels declined approximately 45% with cell age when expressed relative to total RNA (Fig. 1C). When COX I mRNA levels were expressed relative to a nuclear-encoded transcript (α-tubulin) COX I mRNA levels declined by almost 70% (Fig. 1D). Taking into consideration the age-dependent changes in cellular levels of total RNA (Fig. 1A), COX I mRNA losses expressed per microliter of cells approached 95% with age. COX I reductions are likely due to both general declines in transcription compounded with 35% losses in detectible mtDNA template. Other mitochondria transcripts were affected in the same manner as COX I. There was no apparent change in the relationship between full-length and truncated transcripts, as 16S/COX I ratios were unaffected (Fig. 1E). The ratio of COX I to the unprocessed transcripts (Fig. 1E) was also unaffected by cell age, suggesting there is no general defect in primary transcript processing.

3.2. Mitochondrial functional parameters

Previous studies have shown that the total cellular activities of mitochondrial enzyme such as citrate synthase decline by about 50% with age [13]. The low mitochondrial enzyme activities, combined with high levels of hemoglobin preclude further analyses of mitochondrial enzymes on cellular extracts. Consequently, a modification of the nuclear isolation allowed purification of an isolated mitochondrial preparation suitable for analysis of mitochondrial enzyme stoichiometries. No significant differences were found in the activities of three major mitochondrial enzymes involved in energy metabolism (CS, complex I, COX), suggesting that the enzyme stoichiometries are preserved with age (Fig. 2A). Cellular aging is accompanied by a 45% reduction in oxygen consumption. This reduction does not appear to be due to a loss in respiratory capacity as maximal (uncoupled) respiration rates were similar between the oldest and youngest fractions (Fig. 2B). No significant difference was found in the levels of ATP (Fig. 2C), suggesting that old cells adequately meet their energy requirements, despite a lower respiration rate. However, ADP levels in older cells were approximately 58% higher than in the young cells. Accordingly, ATP/ADP ratios were approximately 50% lower than in the young cells (Fig. 2D).

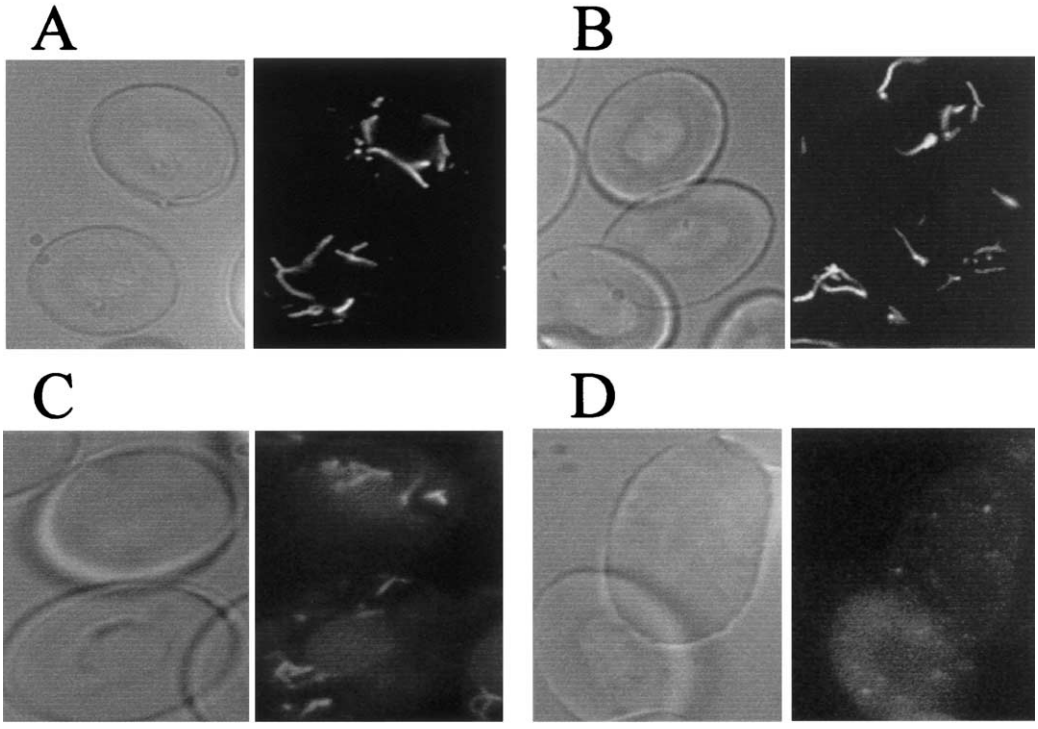

Fig. 3. Fluorescence microscopy of TMRM-loaded erythrocytes treated with mitochondrial permeability transition pore agonists. Each panel shows phase contrast image (left) and fluorescence (right) of erythrocytes loaded with TMRM for 30 min. Cells were incubated in either DMEM (A), with staurosporine (B), cyclosporin A (C) or oligomycin, azide plus dinitrophenol (D).

3.3. Apoptosis

Fluoromicroscopy of mitochondria treated with the ΔΨ-sensitive dye TMRM revealed one to five discrete mitochondria actively moving throughout the cell. Although no quantitative analyses were performed, mitochondria retained fluorescence for at least 1 h. When cells were treated with staurosporine, no depolarization was evident (Fig. 3). In contrast, cells treated with dinitrophenol, azide and oligomycin demonstrated rapid depolarization. Similarly, CsA induced depolarization although not to the extent seen with the mitochondrial inhibitors. In most cases, conditions that led to LDH leakage (an indicator of cell death), also led to DNA fragmentation (an indicator of apoptotic death).

None of the mitochondrial poisons induced apoptosis, since OXPHOS inhibitors and uncouplers in various combinations did not lead to DNA fragmentation (Fig. 4A–C) or LDH leakage or (Fig. 4E–G). Although, oligomycin + dinitrophenol + azide induced mitochondrial depolarization (Fig. 3), it caused no fragmentation (Fig. 4C) or caspase 3 induction (Fig. 4D). Pro-oxidants including sodium nitroprusside (Fig. 4) and paraquat (not shown) failed to induce DNA fragmentation. Although staurosporine typically opens the MPTP in other models [18], it failed to depolarize these mitochondria (Fig. 3) and it did not cause DNA

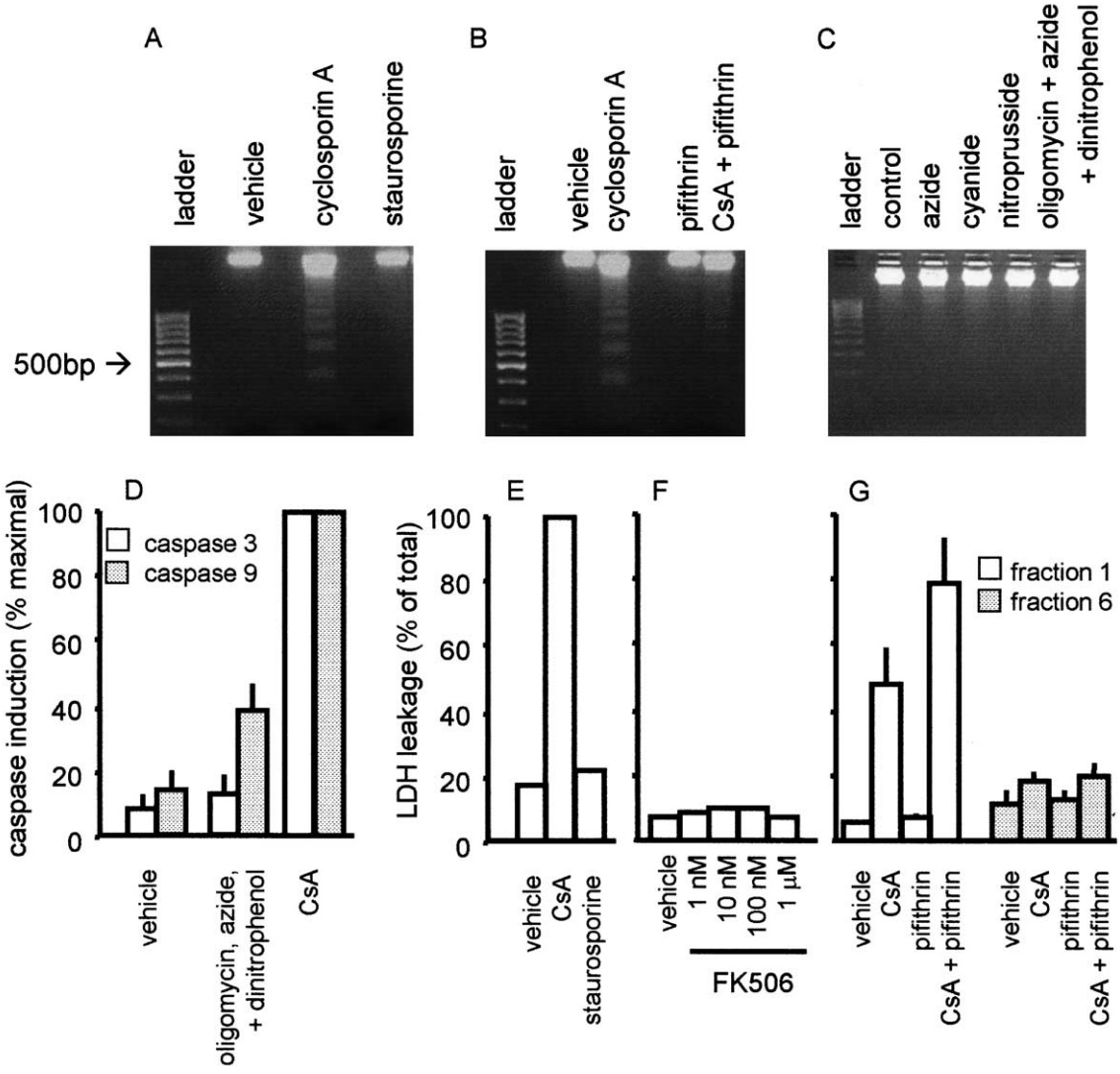

Fig. 4. Apoptotic induction by cyclosporin A. Panels A–C show agarose gels of purified DNA (1–2 μg) from cells treated for 18–24 h. Panel A; the effects of CsA (100 μM) and staurosporine (2 μM). Panel B; pifithrin (200 μM) alone and in combination with CsA. Panel C; mitochondrial inhibitors (10 mM azide, 10 mM cyanide), pro-oxidants (10 mM nitroprusside) and a combination of oligomycin (1 μg/ml), azide (10 mM) and dinitrophenol (0.35 mM). Panel D shows the effects of CsA and staurosporine on caspase 3 and 9 activities (scaled to the maximum values observed with CsA), expressed as mean ($n=4$) with bars indicating S.E. Panels E–F demonstrate LDH leakage from cells undergoing various treatments. The LDH data in Panel E corresponds to the CsA and staurosporine treatments described in Panel A. Panel F examines the effects of FK506 on LDH leakage. Panel G shows the LDH data corresponding to the CsA and pifithrin treatments outlined in Panel B.

fragmentation (Fig. 4A), LDH leakage (Fig. 4E), or activation of either caspase 3 or 9 (Fig. 4D).

CsA is typically protective in apoptotic treatments by virtue of an interaction with a mitochondrial cyclophilin [19]. In these cells, however, CsA induced cell death in trout erythrocytes, based upon DNA fragmentation (Fig. 4A and B), LDH leakage (Fig. 4E) and activation of caspases 3 and 9 (Fig. 4D). The CsA EC_{50} was very high (approximately 40 μM), much higher than that required to protect mitochondria from MPTP agonists (< 1 μM). Only the youngest fraction demonstrated CsA-induced LDH leakage (Fig. 4G) and caspase activation (data not shown).

Other inhibitors of cyclophilin-dependent processes were examined for their ability to mimic or prevent CsA-induced cell death. FK506, an inhibitor of the cyclophilin-dependent phosphatase calcineurin, had no effect on LDH leakage (Fig. 4F). The dose of CsA required to cause cell death was much greater than that required to maximally inhibit calcineurin (< 1 μM). Another potential target for CsA is the cyclophilin dependence of the p53 apoptotic cascade. Pifithrin, a p53 inhibitor, did not induce DNA fragmentation (Fig. 4C) or LDH leakage (Fig. 4G). However, pifithrin exacerbated the effects of CsA on LDH leakage but, again, only in the youngest fraction (Fig. 4G).

Fig. 5 summarizes the effects of a spectrum of caspase inhibitors used to determine the pathway by which CsA induced apoptosis. While caspase inhibitors 1, 2, 6, 9 and 13 failed to protect the cells from CsA-induced DNA fragmentation, inhibitors of caspases 3, 4, 8 and 10 prevented or reduced DNA fragmentation. However, none of the inhibitors prevented LDH leakage in response to CsA.

4. Discussion

4.1. Mitochondrial metabolism and cellular energetics

The role of mitochondria in nucleated fish erythrocytes was expected to be unusual in many respects. In absolute terms, mitochondrial content of these cells is very low relative to more active tissues such as striated muscle, yet they rely primarily upon oxidative metabolism for ATP synthesis [13]. Based upon a number of lines of evidence, it appears as if erythrocyte mitochondria function at a very high proportion of their maximal capacity. Uncoupling young cells using dinitrophenol titrations increased cellular respiration rates only marginally. The basal respiration rate of young erythrocytes (100 nmol O_2/min/g), expressed relative to CS activity (1 U CS/g; [13]) is 100 nmol O_2/min/U CS, a value which exceeds the maximal respiratory rate observed with trout white muscle mitochondria in vitro (60 nmol O_2/min/U CS; [20]). The ATP/ADP ratios seen in young erythrocytes in this study are low enough to drive isolated trout mitochondria at near-maximal rates [20] (this assumes that most of the ADP measured in perchloric acid extracts of erythrocytes is free, in contrast to skeletal muscle, where most of the tissue ADP is bound to myofibrils). Collectively, these data argue that the mitochondrial

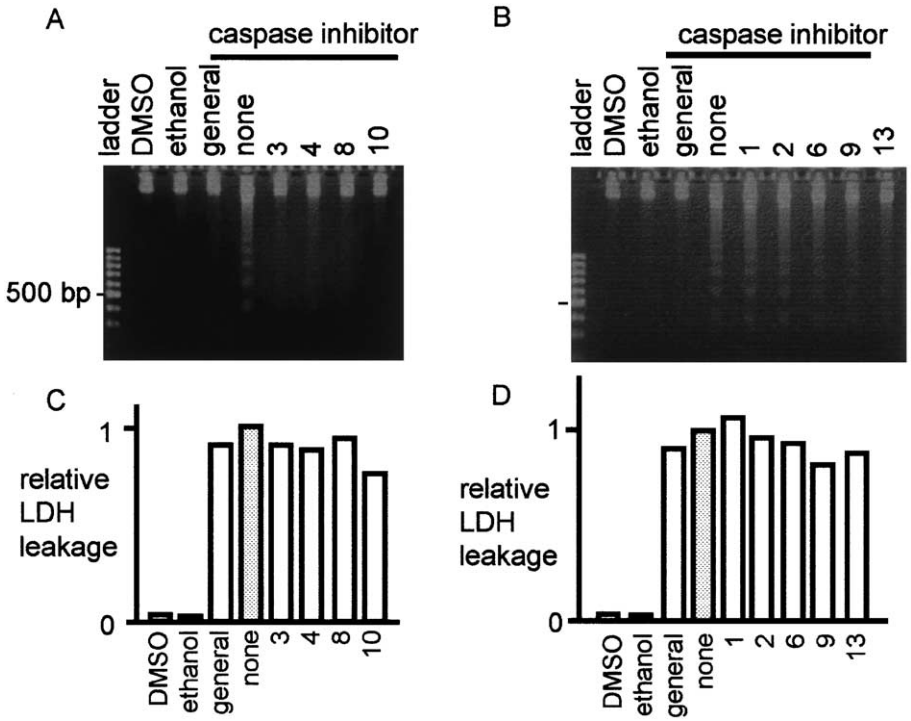

Fig. 5. Effects of caspase inhibitors on CsA-induced cell death. Erythrocytes from whole blood were treated 18 h with 250 μM CsA in the presence of 50 μM caspase inhibitors. Panels A and B are agarose gels of DNA (1 μg) showing DNA laddering. Panels C and D represent LDH leakage from the same cells.

respiration rate of young erythrocytes in vivo is near the maximal rate.

Cellular aging in this trout erythrocyte model leads to 35–50% losses in mtDNA copy number (Fig. 1) and mitochondrial enzymes [13], but it is unclear if these losses compromise cellular energetics. It is likely that erythrocytes can tolerate a loss of mitochondria because of the reduced metabolic demands; although the metabolic costs for ion and gas transport persist throughout the lifetime of the cell, rates of biosynthesis decline precipitously with age [12]. ATP levels are unaffected by cell age, however, there is some evidence that glycolytic production of ATP increases with cell age [7,13]. Nonetheless, significant reserve respiratory capacity exists in older cells, based upon the 2-fold increase in respiration rate upon addition of an uncoupler. Losses of mitochondrial enzymes and mtDNA with aging may reflect metabolic adjustments similar to de-training effects on skeletal muscle [21]. Paradoxically, maximal respiratory rates are similar in young and old cells, despite 30–60% losses in the mitochondrial content, based upon mtDNA levels (Fig. 1) and cellular activities of the marker enzyme citrate synthase [13]. Furthermore, the stoichiometries of the mitochondrial enzymes were largely preserved (Fig. 2A), implying the electron transport enzymes complex I and cytochrome oxidase declined in parallel with citrate synthase. In other systems, electron transport proteins are present in modest excess; inhibition of 35–70% of the activity of complex I [22] and COX [23] can be achieved without impairing respiration. Thus, it is likely that the losses in mitochondrial proteins associated with electron transport can be tolerated without compromising erythrocyte energy metabolism.

4.2. Mitochondrial biogenesis

Studies designed to understand the control of respiratory gene expression typically use diverse models involving induction of mitochondrial proliferation by exercise, electrical stimulation, thyroid hormone, growth hormone and retinoic acid [21]. While it is generally held that mitochondrial transcription reflects the mtDNA copy number [24], more recent studies have shown diverse patterns in both nuclear and mitochondrial gene expression [25]. In this study using erythrocytes as a model of mitochondrial decline, several important differences emerged relative to models of mitochondrial proliferation. It is likely that the most significant influence on mitochondrial gene expression was the global decline in nuclear gene expression.

In trout erythrocytes, aging leads to reductions in global protein synthesis, as indicated by polyribosome abundance [8] and ^{35}S-methionine incorporation [12]. The mRNA levels for proteins involved in gas exchange and acid–base balance decline uniformly with age [12]. The global reduction in transcription and translation is probably related to heme-dependent controls [26]. Reductions in mitochondrial mRNA appear to be due to combined effects of both reduced mtDNA copy number (35%) and global effects on transcription. While we did not quantify potential mtDNA mutations, a common defect in organismal aging models, probing slot blots with cDNA complementary to the common 5 kb deletion yielded the same patterns (data not shown) as probing with the COX I–COX II mtDNA probe. The short lifetime of this cell likely precludes significant accumulation of the sort of deletions that are seen in organismal aging models. Although mtDNA copy number declines, the relationships between truncated vs. full-length transcripts and processed vs. unprocessed transcripts are preserved. We did not determine the relative importance of mtDNA replication control vs. organellar degradation in determining the loss of mtDNA with erythrocyte age.

In many tissues, mitochondria are important sources of ROS, particularly when mitochondrial defects exist. In contrast to other aerobic tissues, mitochondria are probably a minor contributor to oxidative stress in erythrocytes. Cytoplasmic superoxide production is an inevitable consequence of oxygen delivery by hemoglobin. An active methemoglobin reductase regenerates oxidized hemoglobin [15]. Erythrocytes also appear to be an important element of the anti-oxidant defence strategy of tissues. In addition to anti-oxidant enzymes, hemoglobin subunits contain peroxidase activity [27,28]. Erythrocytes protected co-cultured neutrophils from apoptosis induced by oxidative stress in a manner dependent upon catalase activity and glutathione metabolism [29]. Unlike mammalian enucleated erythrocytes, trout erythrocytes maintain anti-oxidant enzyme levels throughout their life. The cellular activities of glutathione peroxidase and catalase increase with age in trout erythrocytes [30]. The activity of superoxide dismutase in trout erythrocytes is unaffected by cell age (unpublished observation). Since mitochondrial levels per cell are relatively low, and with little evidence for mitochondrial dysfunction arising with age, it is unlikely that erythrocyte mitochondria could significantly compromise anti-oxidant defences. While mitochondrial changes during cell aging may not contribute to oxidative stress, mitochondrial membranes and DNA may be targets of ROS damage and accelerate mitochondrial degradation [31]. Given the modest changes in mitochondria that occurred over the 4–6-month life span, it is difficult to establish the relative importance of sustained oxidative stress vs. controlled gene expression in determining the mitochondrial phenotype in aged erythrocytes.

4.3. Mitochondria and apoptosis

In many models, MPTP opening can cause depolarization, metabolite permeability and mitochondrial swelling. Damage to the outer mitochondrial membrane can cause release of cytochrome *c* and activation of caspase 9, leading to apoptosis [1,32]. MPTP opening occurs in response to depolarizing agents, including mitochondrial inhibitors and uncouplers, as well as specific agonists such as staurospor-

ine. In this study, staurosporine, pro-oxidants (paraquat, nitroprusside) and a suite of mitochondrial inhibitors (azide, cyanide, oligomycin, dinitrophenol) failed to induce either apoptosis, as indicated by DNA fragmentation, or any form of cell death, as indicated by LDH release. In particular, the combination of dinitrophenol, azide and oligomycin rapidly depolarized the mitochondrial membrane, yet caused no DNA laddering or induction of caspase 3, even after as much as 48 h exposure. These studies suggest mitochondrial pathways are not capable of triggering apoptosis. It is possible that even prolonged depolarization, as occurs in response to uncouplers, fails to open the MPTP. Alternately, the permeability transition may occur, but the low mitochondrial content of erythrocytes may not release sufficient amounts of pro-apoptotic proteins (e.g., cytochrome *c*) to activate caspase 9.

CsA, a cyclophilin ligand, typically antagonizes the permeability transition protecting the cells from apoptotic agents [2]. However, in trout erythrocytes, CsA was found to induce apoptosis, causing LDH leakage, DNA fragmentation and caspase activation. CsA has been shown to induce apoptosis in other models such as rat hepatocytes [33], although the pathway is not clear. The pathway by which CsA induces apoptosis in erythrocytes was not established. It is important to note that the concentrations of CsA required to induce apoptosis were much higher (25–100 μM) than that required to affect the cyclins of MPTP (~ 1 μM). CsA did in fact cause mitochondrial depolarization, as well as induction of caspase 9. However, mitochondrial depolarization (i.e., via azide, dinitrophenol and oligomycin) in itself is not adequate to induce apoptosis in this model. Furthermore, inhibitors for caspase 9 did not alter nuclear fragmentation by CsA. These results suggest that mitochondrial depolarization and subsequent caspase 9 activation accompany CsA treatment but that these changes are secondary effects of induction of other apoptotic pathways. Although many of the caspase inhibitors prevented DNA fragmentation, none prevented cell death (as indicated by LDH release).

Cyclophilins regulate other pathways that have been implicated in apoptosis. The cyclophilin-dependent phosphatase calcineurin is a potential target of CsA but FK506 (1 nM–1 μM), a cyclophilin-independent inhibitor of calcineurin [34] failed to induce apoptosis. As previously mentioned, the kinetics of CsA-induced cell death are not consistent with effects on calcineurin. CsA is known to interact with at least nine cellular targets, but the affinity for CsA is less understood [35]. This high dose may have resulted in interactions with other targets as well, however, it is doubtful that the agent killed cells by non-specific cytotoxicity because it had no effect on the oldest cells. Through pathways regulated by cyclin-dependent kinases, the multi-functional protein p53 can induce apoptosis in any number of ways including death receptor up-regulation, mitogenic down-regulation and up-regulation of pro-apoptotic Bcl-2 family members [36]. Pifithrin, a direct inhibitor of p53 [37], had no effects on its own but did exacerbate CsA-dependent death. While the specific mechanism of action of CsA was not identified, caspase inhibitor profiles suggest a route involving caspases 3, 4, 8 and 10 but not critically dependent upon caspases 1, 2, 6, 9 or 13. This pattern suggests a membrane receptor-mediated pathway, although neither the nature of the signalling pathway nor the receptor ligands are known at this time. In a related study, Weil et al. [38] suggested that chicken erythrocytes possess a caspase-independent pathway of cell death that is inducible by staurosporine and serum starvation. This pathway was more pronounced in cells derived from day 15 embryos (analogous to young cells), relative to cells collected from adult chickens (analogous to old cells). It is noteworthy that in this study, caspase inhibitors could prevent CsA-induced DNA fragmentation but not cell death (as indicated by LDH release). Nucleated erythrocytes of lower vertebrates apparently possess multiple routes of inducible cell death that differ in many respects from conventional apoptotic pathways.

Collectively, these studies suggest that trout erythrocytes are able to cope with the actively regulated mitochondrial changes associated with cellular aging. The cells maintain their ability to carry out important functions like ATP generation and anti-oxidant defence despite these changes. Age-dependent changes in mitochondria are generally damaging to cellular function, in many cases leading to apoptotic cell death. In neuromuscular models, enzyme losses as little as 20% can manifest as bioenergetic defects [22]. In this fish erythrocyte model, mitochondrial enzymes decline by 60% with age [13], yet exert minimal impact on cellular metabolism. While mitochondrial depolarization triggers cell death in many cell types, a spectrum of inhibitors depolarized mitochondria yet failed to induce apoptotic cell death. Unlike mammalian erythrocytes, trout nucleated erythrocytes retain the capacity for apoptosis, probably via a ligand-mediated route, involving p53.

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References

[1] E.A. Slee, M.T. Harte, R.M. Kluck, B.B. Wolf, C.A. Casiano, D.D. Newmeyer, H.-G. Wang, J.C. Reed, D.W. Nicholson, E.S. Alnemri, D.R. Green, S.J. Martin, Ordering the cytochrome *c*-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependant manner, J. Cell Biol. 144 (1999) 281–292.

[2] J.J. Lemasters, A.L. Nieminen, T. Qian, L.C. Trost, S.P. Elmore, Y. Nishimura, R.A. Crowe, W.E. Cascio, C.A. Bradham, D.A. Brenner, B. Herman, The mitochondrial permeability transition in cell death: a

common mechanism in necrosis, apoptosis and autophagy, Biochim. Biophys. Acta 1366 (1998) 177–196.
[3] C. Turner, A.H. Schapira, Mitochondrial dysfunction in neurodegenerative disorders and ageing, Adv. Exp. Med. Biol. 487 (2001) 229–251.
[4] C.D. Moyes, B.J. Battersby, S.C. Leary, Regulation of muscle mitochondrial design, J. Exp. Biol. 201 (1998) 299–307.
[5] J.E. Keen, A.M. Steele, A.H. Houston, The circulating erythrocytes of rainbow trout (*Salmo gairdneri*), Comp. Biochem. Physiol. 94A (1989) 699–711.
[6] M. Nikinmaa, Vertebrate Red Blood Cells, Springer-Verlag, Berlin, 1990.
[7] H.C. Lane, Nucleoside triphosphate changes during the peripheral life-span of erythrocytes of adult rainbow trout (*Salmo gairdneri*), J. Exp. Zool. 231 (1984) 57–62.
[8] H.C. Lane, J.W. Weaver, J.A. Benson, H.A. Nichols, Some age related changes of adult rainbow trout, *Salmo gairdneri* Rich.; peripheral erythrocytes separated by velocity sedimentation at unit gravity, J. Fish Biol. 21 (1982) 1–13.
[9] W. Speckner, J.F. Schindler, C. Albers, Age-dependent changes in volume and haemoglobin content of erythrocytes in the carp (*Cyprinus carpio* L.), J. Exp. Biol. 141 (1989) 133–149.
[10] L. Tiano, D. Fedeli, P. Ballarini, G. Santoni, G. Falcioni, Mitochondrial membrane potential in density-separated trout erythrocytes exposed to oxidative stress in vitro, Biochim. Biophys. Acta 1505 (2001) 226–237.
[11] L. Tiano, P. Ballarini, G. Santoni, M. Wozniak, G. Falcioni, Morphological and functional changes in mitochondria from density separated trout erythrocytes, Biochim. Biophys. Acta 1457 (2000) 118–128.
[12] S.G. Lund, M.C. Phillips, C.D. Moyes, B.L. Tufts, The effects of cell ageing on protein synthesis in rainbow trout (*Oncorhynchus mykiss*) red blood cells, J. Exp. Biol. 203 (2000) 2219–2228.
[13] M.C. Phillips, C.D. Moyes, B.L. Tufts, The effects of cell aging on metabolism in rainbow trout (*Oncorhynchus mykiss*) red blood cells, J. Exp. Biol. 203 (2000) 1039–1045.
[14] R. De Maria, A. Zeuner, A. Eramo, C. Domenichelli, D. Bonci, F. Grignani, S.M. Srinivasula, E.S. Alnemri, U. Testa, C. Peschle, Negative regulation of erythropoiesis by caspase-mediated cleavage of GATA-1, Nature 401 (1999) 489–493.
[15] D. Bratosin, J. Mazurier, J.P. Tissier, J. Estaquier, J.J. Huart, J.C. Ameisen, D. Aminoff, J. Montreuil, Cellular and molecular mechanisms of senescent erythrocyte phagocytosis by macrophages. A review, Biochimica 80 (1998) 173–195.
[16] B.J. Battersby, C.D. Moyes, Influence of acclimation temperature on mitochondrial DNA, RNA, and enzymes in skeletal muscle, Am. J. Physiol. 275 (1998) R905–R912.
[17] C.D. Moyes, O.A. Mathieu-Costello, N. Tsuchiya, C. Filburn, R.G. Hansford, Mitochondrial biogenesis during cellular differentiation, Am. J. Physiol. 272 (1997) C1345–C1351.
[18] E. Daugas, S.A. Susin, N. Zamzami, K.F. Ferri, T. Irinopoulou, N. Larochette, M.C. Prevost, B. Leber, D. Andrews, J. Penninger, G. Kroemer, Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis, FASEB J. 14 (2000) 729–739.
[19] T. Qian, B. Herman, J.J. Lemasters, The mitochondrial permeability transition mediates both necrotic and apoptotic death of hepatocytes exposed to Br-A23187, Toxicol. Appl. Pharmacol. 154 (1999) 117–125.
[20] C.D. Moyes, P.M. Schulte, P.W. Hochachka, Recovery metabolism of trout white muscle: role of mitochondria, Am. J. Physiol. 262 (1992) R295–R304.
[21] D.A. Hood, Contractile activity-induced mitochondrial biogenesis in skeletal muscle, J. Appl. Physiol. 90 (2001) 1137–1157.
[22] A. Barrientos, C.T. Moraes, Titrating the effects of mitochondrial complex I impairment in the cell physiology, J. Biol. Chem. 274 (1999) 16188–16197.
[23] S.C. Leary, B.J. Battersby, R.G. Hansford, C.D. Moyes, Interactions between bioenergetics and mitochondrial biogenesis, Biochim. Biophys. Acta 1365 (1998) 522–530.
[24] R.S. Williams, Mitochondrial gene expression in mammalian striated muscle, J. Biol. Chem. 261 (1986) 12390–12394.
[25] N. Lenka, C. Vijayasarathy, J. Mullick, N.G. Avadhani, Structural organization and transcription regulation of nuclear genes encoding the mammalian cytochrome *c* oxidase complex, Prog. Nucleic Acid Res. Mol. Biol. 61 (1998) 309–344.
[26] O. Fuchs, A. Hradilek, J. Borova, J. Neuwirt, M. Travnicek, Effect of heme on globin messenger RNA synthesis, Acta Biol. Med. Ger. 40 (1981) 915–925.
[27] R. Gabbianelli, A.M. Santroni, D. Fedeli, A. Kantar, G. Falcioni, Antioxidant activities of different hemoglobin derivatives, Biochem. Biophys. Res. Commun. 242 (1998) 560–564.
[28] D. Fedeli, L. Tiano, R. Gabbianelli, G.C. Caulini, M. Wozniak, G. Falcioni, Hemoglobin components from trout (*Salmo irideus*): determination of their peroxidative activity, Comp. Biochem. Physiol., Part B Biochem. Mol. Biol. 130 (2001) 559–564.
[29] K. Aoshiba, Y. Nakajima, S. Yasui, J. Tamaoki, A. Nagai, Red blood cells inhibit apoptosis of human neutrophils, Blood 93 (1999) 4006–4010.
[30] G. Falcioni, F. Grelloni, A.R. Bonfigli, E. Bertoli, Biochemical characterization of density-separated trout erythrocytes, Biochem. Int. 28 (1992) 379–384.
[31] V.N. Luzikov, Quality control: from molecule to organelles, FEBS Lett. 448 (1999) 201–205.
[32] S.A. Susin, N. Zamzami, G. Kroemer, Mitochondria as regulators of apoptosis: doubt no more, Biochim. Biophys. Acta 1366 (1998) 151–165.
[33] S. Grub, E. Persohn, W.E. Trommer, A. Wolf, Mechanisms of cyclosporin A-induced apoptosis in rat hepatocyte primary cultures, Toxicol. Appl. Pharmacol. 163 (2000) 209–220.
[34] F. Rusnak, P. Mertz, Calcineurin: form and function, Physiol. Rev. 80 (2000) 1483–1521.
[35] M. Crompton, The mitochondrial permeability transition pore and its role in cell death, Biochem. J. 341 (1999) 233–249.
[36] M.S. Sheikh, A.J.J. Fornace, Role of p53 family members in apoptosis, J. Cell. Physiol. 182 (2000) 171–181.
[37] P.G. Komarov, E.A. Komarova, R.V. Kondratov, K. Christov-Tselkov, J.S. Coon, M.V. Chernov, A.V. Gudkov, A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy, Science 285 (1999) 1733–1737.
[38] M. Weil, M.D. Jacobson, M.C. Raff, Are caspases involved in the death of cells with a transcriptionally inactive nucleus? Sperm and chicken erythrocytes, J. Cell Sci. 111 (1998) 2707–2715.